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# Monitoring of Human Herpesvirus-6 and -7 Genomes in Saliva Samples of Healthy Adults by Competitive Quantitative PCR

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Human herpesviruses-6 and -7 (HHV-6 and HHV-7) are thought to be transmitted during early infancy through saliva. However, the kinetics of the virus shedding in saliva of healthy adults, from whom children are assumed to acquire the viruses, is mostly unknown. This study was conducted to determine how many copies of the genome are secreted in saliva of healthy adults and to clarify the relationship between viral DNA load and virus isolation of HHV-6 and HHV-7. Competitive PCR was performed using primer sets in the U42 gene of each viral genome. In saliva samples from 29 healthy adults, HHV-6 and HHV-7 DNA was detected in 41.4% and 89.7%, respectively. The average copy number of the HHV-7 genome in the positive samples was higher than that of the HHV-6 genome. Follow-up studies of six seropositive individuals for 3 months showed that the amount of HHV-7 DNA was constant in each individual and that "high producers" and "low producers" could be distinguished. By contrast, the amount of HHV-6 DNA varied drastically over time in each individual. Although HHV-6 was never isolated from the saliva of any of the six individuals during the follow-up period, HHV-7 was isolated from each individual several times. The amount of HHV-7 DNA tended to be higher at the times when the virus was isolated than at the times when the virus was not isolated. These data demonstrate a striking contrast between HHV-6 and HHV-7 in the kinetics of genome and virus shedding. ***J. Med. Virol. 61:208–213, 2000.*** 

**KEY WORDS:** quantitative PCR; HHV-6; HHV-7; saliva; virus shedding; route of transmission

## INTRODUCTION

Human herpesvirus-6 (HHV-6) was first isolated from peripheral blood lymphocytes of patients with lymphoproliferative disorders [Salahuddin et al., 1986]. HHV-6 is a causative agent of roseola (exanthema subitum), a febrile childhood disease accompanied by rash [Yamanishi et al., 1988]. Human herpesvirus-7 (HHV-7) was first isolated from activated peripheral blood lymphocytes of a healthy person [Frenkel et al., 1990] and also causes roseola [Tanaka et al., 1994]. HHV-6 and HHV-7 exhibit considerable genomic sequence homology and are classified as members of the *Roseolavirus* genus of the *Betaherpesvirinae* subfamily [Gompels et al., 1995; Nicholas 1996]. Like the other members of *Herpesviridae*, HHV-6 and HHV-7 are thought to establish latent, lifelong infection after the primary infection [Hidaka et al., 1993; Tanaka et al., 1996].

HHV-6 and HHV-7 are believed to be transmitted during early infancy by saliva. However, the kinetics of the virus shedding in the saliva of healthy adults, from whom children are assumed to acquire the viruses, is mostly unknown. HHV-7 is isolated more readily from the saliva of healthy adults than is HHV-6 [Black et al., 1993; Hidaka et al., 1993; Takahashi et al., 1997; Wyatt and Frenkel 1992]. Seroconversion of HHV-6 occurs commonly before 1.5 years of age, a few years earlier than that of HHV-7 infection [Tanaka et al., 1996; Wyatt et al., 1991].

The aims of this study were to determine how many copies of the HHV-6 and HHV-7 genomes are secreted in the saliva of healthy adults and to clarify the relationship between the copy number of the genomes and the presence of infectious HHV-6 and HHV-7.

## MATERIALS AND METHODS

### PCR Primers for QT-PCR

Quantitative polymerase chain reaction (QT-PCR) assays were carried out for HHV-6 and HHV-7 accord-

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ing to a previous report [Secchiero et al., 1995] with several modifications. Specific primers were constructed to amplify a part of the U42 gene of each virus. The primer pair for HHV-6 delineating 260 base pairs (bp) (map pos.; 69078–69337) was 642F(24 mer, 5′-GTT TAA GTT AGT TTC ACA GGT GTC-3′) and 642R (22 mer, 5′-CCA AAG TTT GCG TCG AAA AAC C-3′), and that for HHV-7 delineating 218 bp (map pos., 62800-63017) was 742F (23 mer, 5′-TTA ATG TCG GTC TCA CAA GTT TC-3′) and 742R (23 mer, 5′-AAC AGA CTG AAA AAA GAC TGG AG-3′).

### Construction of Competitors

Competitor DNA was constructed by PCR using a Competitive DNA Construction Kit according to the manufacturer's protocol (TaKaRa, Tokyo, Japan). The λ phage sequence was used to construct competitors in the kit, although the pGEM4Z plasmid sequence was used in the original method [Secchiero et al., 1995]. To ensure the comparative efficiency of amplification of the target DNA and its competitor, the length of the competitor was designed to be less than 120% of the target. For this purpose, specific primer pairs were constructed, i.e., 642F+F (5′-GTT TAA GTT AGT TTC ACA GGT GTC gta cgg tca tca tct gac-3′) and 642R+250R (5′-CCA AAG TTT GCG TCG AAA AAC Cag agt ttc tgc ggc agt taa-3′) to amplify a 296-bp competitor for HHV-6, and 742F+F (5′-TTA ATG TCG GTC TCA CAA GTT TCg tac ggt cat cat ctg aca c-3′) and 742R+200R (5′-AAC AGA CTG AAA AAA GAC TGG AGt cat tac gca tcg cta tta-3′) to amplify a 246-bp competitor for HHV-7. The 3′ half (shown in lower case) of each primer represents the λ DNA sequence and served for amplification of competitor DNA, while the 5′ half (underlined) was identical to that of the corresponding primer described above and served for co-amplification of target DNA in the later step. The amplification reaction was carried out by adding template λ DNA to a reaction mixture containing 10 mM Tris-HCl (pH 8.3, 25°C), 50 mM KCl, 1.5 mM $MgCl_2$, 400 nM of each primer, 200 M each dNTP, and 1.25 U of Ex Taq DNA Polymerase (TaKaRa). Thermal cycling conditions were as follows: 1 cycle at 94°C for 1 min; 30 cycles at 94°C for 30 sec, 60°C for 30 sec, and at 72°C for 30 sec; 1 cycle at 72°C for 5 min. Amplified competitor DNA was purified with a spin column (SUPREC-02, TaKaRa). The number of DNA copies was determined by measuring $A_{260}$ with a spectrophotometer.

### Saliva Samples

After informed consent was obtained, saliva samples were collected from 29 healthy adults for detection of the HHV-6 and HHV-7 genomes. These subjects included 15 males (range, 25–60 years old [y.o.]; average 33 y.o.) and 14 females (range, 21–55 y.o.; average, 32 y.o.). Sequential saliva samples were collected from 6 out of the 29 healthy adults over a 3-month period. Samples were processed within a day for virus isolation, or stored at −80°C until used for QT-PCR.

### Extraction of DNA from Saliva Samples

Saliva samples were boiled for 10 min and centrifuged at 7,000 rpm (2,000*g*) for 3 min. The supernatant (400 μl) was collected. DNA was extracted with a GeneClean II Kit (BIO 101 Inc., Vista, CA) and finally dissolved in 40 μl TE buffer.

### PCR Reaction Conditions

Each PCR reaction was composed of 10 mM Tris-HCl (pH 8.3, 25°C), 50 mM KCl, 1.5 mM $MgCl_2$, 400 nM of each primer, 200 μM each dNTP, and 1.25 U of AmpliTaq Gold DNA Polymerase (Perkin-Elmer Cetus, Foster City, CA). For QT-PCR, thermal cycling conditions were as follows: 1 cycle at 95°C for 9 min; 45 cycles at 60°C for 45 sec, and at 94°C for 30 sec; 1 cycle at 60°C for 5 min.

### Control Target DNA for Standard Curves

To generate standard curves, control target DNA was generated by PCR cloning. HHV-6 (HST strain) and HHV-7 (MSO strain) DNA was amplified, ligated into the pGEM-T easy vector (Promega, Madison, WI), and used to transform competent *Escherichia coli*. The plasmid DNA of each transformant was purified with a QIAprep Spin Miniprep Kit according to the manufacturer's protocol (QIAGEN, Valencia, CA). The number of DNA copies was determined by measuring $A_{260}$ with a spectrophotometer.

### Analysis of Clinical Samples by QT-PCR

To measure the viral DNA load, a series of QT-PCRs were carried out. In this investigation, 2μl of each DNA sample, corresponding to 20 μl of the initial saliva sample, was subjected to PCR with a known copy number of the competitor DNA. After the competitive PCR, the products were separated by gel electrophoresis in 3% low-melting-point agarose (NuSieve, FMC BioProducts, Rockland, ME) and stained with GelStar Nucleic Acid Gel Stain (TaKaRa). The densities of sample and competitor bands were measured with a FluorImager (Molecular Dynamics, Sunnyvale, CA) and the number of copies of viral genome in each sample was calculated from the ratio of the densities.

### Isolation of HHV-6 and HHV-7 from Saliva Samples

HHV-6 and HHV-7 were isolated from saliva as described previously [Takahashi et al., 1997]. Briefly, about 3 ml of saliva was diluted 1:1 with RPMI 1640 medium. After low-speed centrifugation, the supernatant was filtered through a 0.45-μm Millex-HA filter (Millipore, Bedford, MA). Five million human cord blood mononuclear cells stimulated with 0.1 U/ml recombinant interleukin-2 (IL-2) and 5 μg/ml phytohemagglutinin (PHA) were inoculated with 5 ml of each saliva sample and centrifuged at 3,000 rpm (1,000*g*) for 60 min to enhance adsorption. The cell pellets were resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 0.1 U/ml IL-2 and 5

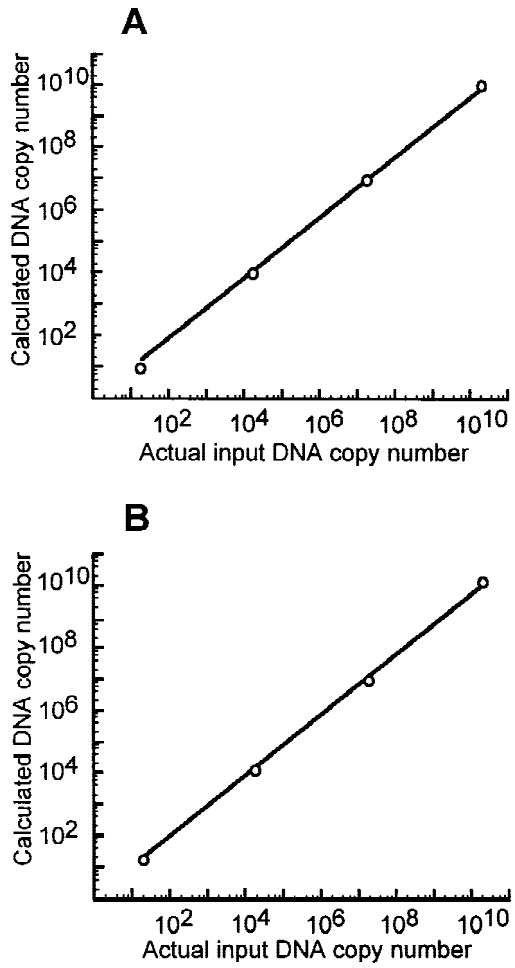


Fig. 1. Calibration curves of quantitative polymerase chain reaction (QT-PCR) for human herpesvirus-6 (HHV-6) (**A**) and human herpesvirus-7 (HHV-7) (**B**). Known copy numbers of control DNA (four concentrations from 20 to $2 \times 10^{10}$ molecules) were co-amplified with competitors in the QT-PCR procedure. Calculated target DNA copy numbers were plotted against actual input copy numbers. Each point represents the mean of triplicate determinations. The lines of best fit were determined by the least-squares method.

μg/ml phytohemagglutinin, and maintained for 4 weeks. Identification of the virus isolates was done by immunofluorescence (IF) using monoclonal antibody TK-2 directed to HHV-6 and IK-3 to HHV-7 [Tsukazaki et al., 1998]. Samples that did not exhibit either cytopathic changes or positive immunofluorescence by 4 weeks postinoculation were considered negative for virus isolation.

## RESULTS

### Calibration Curves of QT-PCR

To assess the performance of QT-PCR, calibration curves were constructed for each virus (Fig. 1). There was a strong correlation between the actual input DNA copy and calculated copy numbers, with correlation coefficients $r^2$ of 0.9994 for HHV-6 and 0.9983 for HHV-7. The assays for both HHV-6 and HHV-7 have broad dynamic ranges with a detection limit of approximately

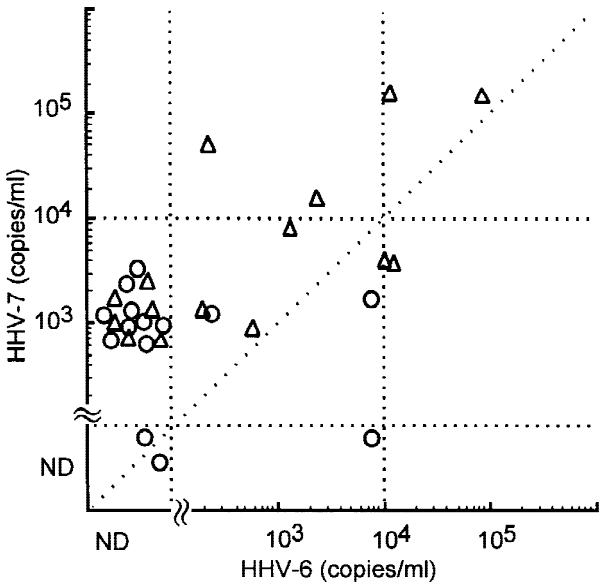


Fig. 2. Number of copies of human herpesvirus-7 (HHV-7) genome plotted in relation to the number of copies of HHV-6 genome (logarithmic scale) in saliva samples obtained from 29 healthy adults, i.e., 15 males (△) and 14 females (○). ND, not detected, i.e., below detection limit.

10 DNA copies, and the actual detection limit in the saliva samples was estimated at approximately 200 DNA copies/ml of saliva. Based on the similar correlation coefficients for HHV-6 and HHV-7 and the similar PCR signals for these viruses at the minimum dilution point, the efficiency of QT-PCR for these viruses was judged to be comparable. No cross-reactive amplification between HHV-6 and HHV-7 was observed under the experimental conditions in which the heterologous targets were present. No cross-reactive amplification with other herpesviruses (herpes simplex virus types 1 and 2, cytomegalovirus, Epstein-Barr virus) was observed.

### DNA Copy Numbers of HHV-6 and HHV-7 in Saliva Samples

Figure 2 shows the relationship between the number of copies of HHV-6 genome and the number of copies of HHV-7 genome in saliva samples determined by the QT-PCR procedure. The genomes of HHV-6 and HHV-7 were detected in 41.3% (12/29) and 89.7% (26/29) of the saliva samples from 29 healthy adults. The average DNA copy number of the HHV-6 genome in the positive samples ($4.8 \times 10^3$ copies/ml) was much lower than that of the HHV-7 genome ($1.4 \times 10^4$ copies/ml)($P < 0.05$, Mann-Whitney U-test). Only 4 of 29 individuals showed more HHV-6 DNA than HHV-7 DNA. More than one-half of the samples (17/29; 6/15 males and 11/14 females) showed a distribution in which the amounts of HHV-6 DNA were below the detection limit and the amounts of HHV-7 DNA ranged below $5 \times 10^3$ copies/ml. Saliva samples of 6 males contained more than $1.0 \times 10^4$ copies/ml of either the HHV-6 or HHV-7

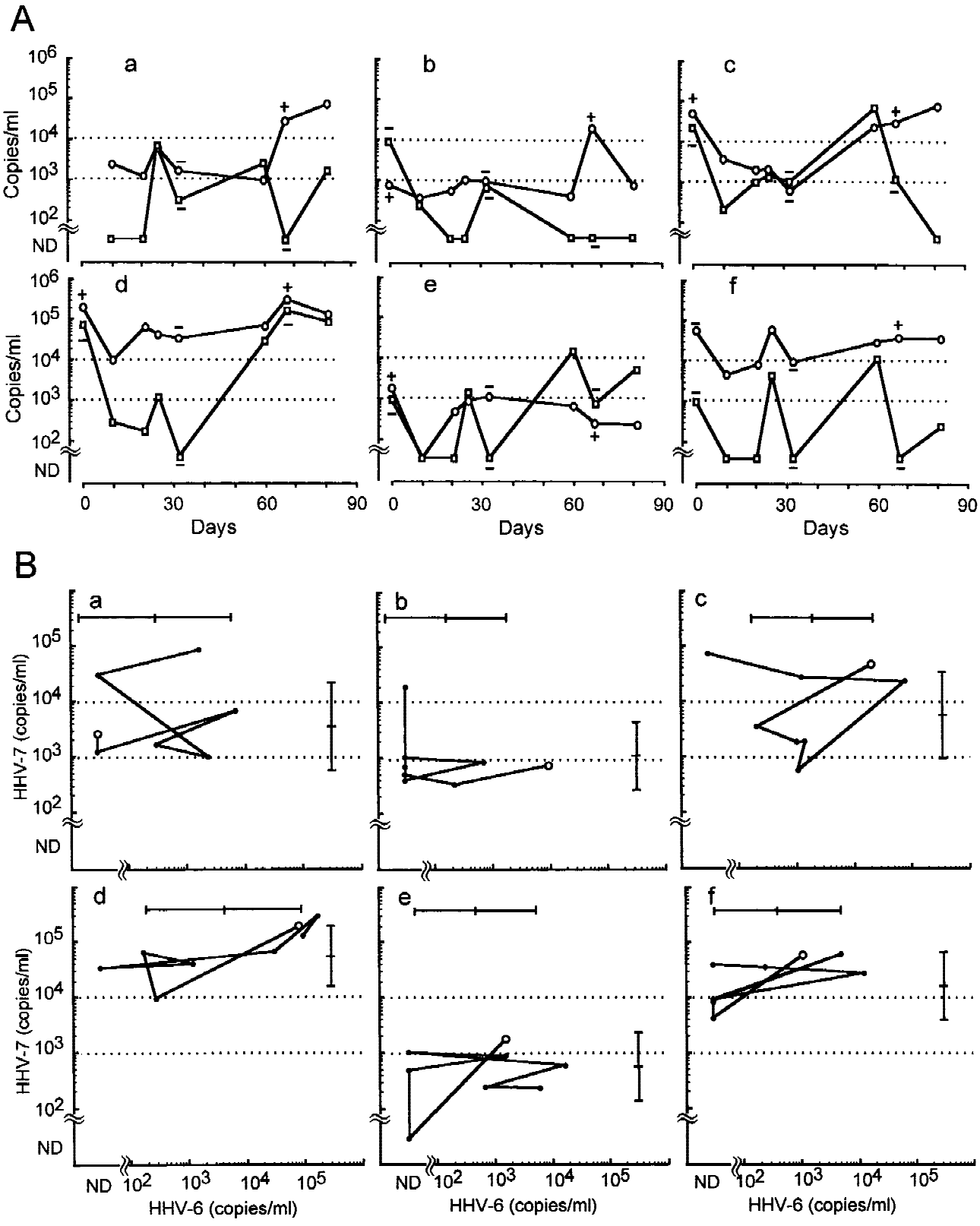


Fig. 3. **A:** Kinetics of copy numbers (logarithmic scale) of the human herpesvirus-6 (HHV-6) genome (□) and the HHV-7 genome (○) in saliva samples of six seropositive healthy adults during a 3-month period. The results of virus isolation are shown beside the points at which virus isolation was attempted. **B:** Number of copies of the HHV-7 genome plotted in relation to the number of copies of HHV-6 (logarithmic scales) in saliva samples of the six individuals. For each subject, the first point is shown with a large circle and the points are then connected in chronological order. Error bars, mean copy numbers with standard deviation. ND, not detected, i.e., below detection limit.

TABLE I. Profiles of the Six Healthy Adults Whose Saliva Samples Were Monitored for the HHV-6 and HHV-7 Genomes During a 3-Month Period

| | | | HHV-6 | | HHV-7 | |
|---|---|---|---|---|---|---|
| Subject | Age | Sex | Mean copy no. of the genome | Isolation rate from saliva | Mean copy no. of the genome | Isolation rate from saliva |
| a | 32 | M | $3.3 \times 10^3$ | 0/2 | $4.1 \times 10^3$ | 1/2 |
| b | 46 | M | $1.7 \times 10^3$ | 0/3 | $1.1 \times 10^3$ | 2/3 |
| c | 28 | M | $1.6 \times 10^3$ | 0/3 | $6.0 \times 10^3$ | 2/3 |
| d | 27 | M | $4.5 \times 10^3$ | 0/3 | $5.6 \times 10^4$ | 2/3 |
| e | 42 | M | $1.9 \times 10^3$ | 0/3 | $5.1 \times 10^2$ | 2/3 |
| f | 44 | F | $2.3 \times 10^3$ | 0/3 | $1.7 \times 10^4$ | 1/3 |

genomes, 2 of which contained more than $1.0 \times 10^4$ copies/ml of both genomes.

### Kinetics of Viral DNA Copy Numbers in Saliva Samples

The virus DNA load of sequential samples from 6 healthy adults was monitored for 3 months (Fig. 3A). The relationship between the number of copies of the two genomes is shown in Figure 3B. The amount of HHV-7 DNA was rather constant in each individual. Some individuals (d and f) were "high-producers"; others (b and e) were "low producers" of HHV-7 DNA. By contrast, the amount of HHV-6 DNA varied drastically over time in each individual, from below detection limit to more than $1.0 \times 10^5$ copies/ml. In subjects a and c, the amounts of both HHV-6 and HHV-7 DNA were extremely variable.

### Relationship Between DNA Amounts in Saliva Samples and Virus Isolation Rates

During the 3-month period, HHV-6 was never isolated from the saliva of any of the 6 individuals, while HHV-7 was isolated from all of the 6 individuals at several times (Fig. 3A and Table I). The amounts of HHV-7 DNA tended to be higher at the times when the isolation was positive than at the times when the isolation was negative in each individual. However, one of the "high producers" (subject f) exhibited an isolation rate of 1/3, while both of the "low producers" (subjects b and e) exhibited isolation rates of 2/3, suggesting that DNA copy numbers and isolation rates of HHV-7 were not correlated completely.

Figure 4 shows the viral DNA load of saliva samples from which virus isolation was positive and negative. A total of 29 saliva samples from the 6 individuals including 12 samples obtained after the 3-month period were plotted. The average copy number of HHV-7 DNA seemed to be higher at the times at which the isolation was positive ($9.5 \times 10^3$ copies/ml) than at the times when the isolation was negative ($2.6 \times 10^3$ copies/ml), although the statistic significance was not shown ($P = 0.0723$). HHV-6 was not isolated even when DNA copy numbers of HHV-6 were more than $1 \times 10^5$ copies/ml, comparable to the maximum DNA copy numbers of HHV-7.

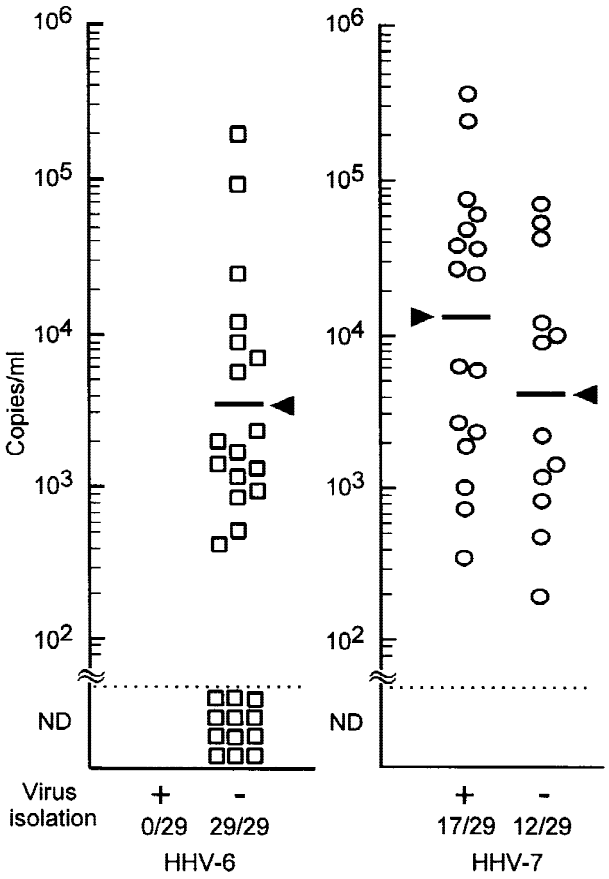


Fig. 4. Copy numbers of the human herpesvirus-6 (HHV-6) and HHV-7 genomes (logarithmic scales) in saliva samples, from which virus isolation was positive (+) or negative (–). Arrowheads, mean values. ND, not detected, i.e., below detection limit.

## DISCUSSION

In this study, QT- PCR was employed to determine the number of copies of the HHV-6 and HHV-7 genomes in saliva samples from 29 healthy subjects. The QT-PCR primers on parts of the U42 genes where the nucleotide homology between these viruses is low were selected. In fact, no cross-reactive amplification of heterologous sequence was observed. This specific amplification enabled us to determine the amounts of DNA of each virus in the presence of heterologous viruses in the saliva samples.

In a previous study, horizontal transmission of HHV-7 from parents to children through close contact within a household was reported [Takahashi et al., 1997]. In this study, special attention was focused on two points relevant to the mode of transmission of

these viruses. First, the amounts of DNA of HHV-6 and HHV-7 in saliva samples were simultaneously monitored and a difference between HHV-6 and HHV-7 in the kinetics of the viral DNA load was clearly demonstrated. The cross-sectional profiles of 29 healthy adults and the follow-up of the kinetics of 6 individuals for 3 months demonstrated that the amount of HHV-7 DNA was rather constant for each individual and that "high producers" and "low producers" could be distinguished. By contrast, the amount of HHV-6 DNA varied drastically over time in each individual. Kidd et al. [1996] reported the measurement of HHV-7 DNA in peripheral blood and saliva of healthy subjects by QT-PCR. Although their observation period was limited (25 days), they also reported a sustained virus load of HHV-7 in each saliva sample.

Second, the relationship between the amounts of viral DNA and the virus isolation rates was examined. Although HHV-6 was never isolated from the saliva of any of the individuals, HHV-7 was isolated from all six of the individuals at several times during the follow-up period. The failure to isolate HHV-6 from the saliva samples cannot be attributed to the scarcity of the amounts of HHV-6 DNA, because at several times the amounts of HHV-6 DNA were almost comparable to the maximum amount of HHV-7 DNA. Therefore, the enigma as to why HHV-6 infection usually precedes HHV-7 infection by a few years during early childhood still remains to be resolved. One possibility is that HHV-6 is usually present in saliva in a noninfectious form by binding to some inhibitory factors and that at some special occasions HHV-6 becomes infectious. In this context, it would be interesting to examine the neutralising activity of secretory antibodies in saliva. The other possibility is that HHV-6 might be transmitted by routes other than saliva. Detection of HHV-6 DNA from genital secretions has been reported [Okuno et al., 1995], and further studies should be conducted to investigate the possibility of perinatal infection.

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## REFERENCES

Black J, Inoue N, Kite Powell K, Zaki S, Pellett P. 1993. Frequent isolation of human herpesvirus 7 from saliva. Virus Res 29:91–98.

Frenkel N, Schirmer E, Wyatt L, Katsafanas G, Roffman E, Danovich R, June C. 1990. Isolation of a new herpesvirus from human $CD4^+$ T cells. Proc Natl Acad Sci USA 87:748–752.

Gompels U, Nicholas J, Lawrence G, Jones M, Thomson B, Martin M, Efstathiou S, Craxton M, Macaulay H. 1995. The DNA sequence of human herpesvirus-6: structure, coding content, and genome evolution. Virology 209:29–51.

Hidaka Y, Liu Y, Yamamoto M, Mori R, Miyazaki C, Kusuhara K, Okada K, Ueda K. 1993. Frequent isolation of human herpesvirus 7 from saliva samples. J Med Virol 40:343–346.

Kidd I, Clark D, Ait KM, Griffiths P, Emery V. 1996. Measurement of human herpesvirus 7 load in peripheral blood and saliva of healthy subjects by quantitative polymerase chain reaction. J Infect Dis 174:396–401.

Nicholas J. 1996. Determination and analysis of the complete nucleotide sequence of human herpesvirus. J Virol 70:5975–5989.

Okuno T, Oishi H, Hayashi K, Nonogaki M, Tanaka K, Yamanishi K. 1995. Human herpesviruses 6 and 7 in cervixes of pregnant women. J Clin Microbiol 33:1968–1970.

Salahuddin S, Ablashi D, Markham P, Josephs S, Sturzenegger S, Kaplan M, Halligan G, Biberfeld P, Wong SF, Kramarsky B. 1986. Isolation of a new virus, HBLV, in patients with lymphoproliferative disorders. Science 234:596–601.

Secchiero P, Zella D, Crowley R, Gallo R, Lusso P. 1995. Quantitative PCR for human herpesviruses 6 and 7. J Clin Microbiol 33:2124–2130.

Takahashi Y, Yamada M, Nakamura J, Tsukazaki T, Padilla J, Kitamura T, Yoshida M, Nii S. 1997. Transmission of human herpesvirus 7 through multigenerational families in the same household. Pediatr Infect Dis J 16:975–978.

Tanaka K, Kondo T, Torigoe S, Okada S, Mukai T, Yamanishi K. 1994. Human herpesvirus 7: another causal agent for roseola (exanthem subitum). J Pediatr 125:1–5.

Tanaka TK, Kondo T, Mukai T, Miyoshi H, Yamamoto Y, Okada S, Yamanishi K. 1996. Seroepidemiological study of human herpesvirus-6 and -7 in children of different ages and detection of these two viruses in throat swabs by polymerase chain reaction. J Med Virol 48:88–94.

Tsukazaki T, Yoshida M, Namba H, Yamada M, Shimizu N, Nii S. 1998. Development of a dot blot neutralising assay for HHV-6 and HHV-7 using specific monoclonal antibodies. J Virol Methods 73: 141–149.

Wyatt L, Frenkel N. 1992. Human herpesvirus 7 is a constitutive inhabitant of adult human saliva. J Virol 66:3206–3209.

Wyatt L, Rodriguez W, Balachandran N, Frenkel N. 1991. Human herpesvirus 7: antigenic properties and prevalence in children and adults. J Virol 65:6260–6205.

Yamanishi K, Okuno T, Shiraki K, Takahashi M, Kondo T, Asano Y, Kurata T. 1988. Identification of human herpesvirus-6 as a causal agent for exanthem subitum. Lancet 1:1065–1067.